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Research paper

Identification, incidence and control of bacterial blotch disease in mushroom crops by management of environmental conditions

María J. Navarro[a,*], Francisco J. Gea[a], Ana J. González[b]

[a] Centro de Investigación, Experimentación y Servicios del Champiñón (CIES), E-16220 Quintanar del Rey, Cuenca, Spain
[b] Programa de Patología Vegetal, Servicio Regional deInvestigación y Desarrollo Agroalimentario (SERIDA), Carretera de Oviedo s/n. 33300 Villaviciosa, Asturias, Spain

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ABSTRACT

Bacterial brown blotch disease is one of the most serious bacterial diseases in cultivated mushrooms. Fluorescent pseudomonas isolated from different lesions on the caps of cultivated *Agaricusbisporus* were identified as strains of *Pseudomonas tolaasii* and the poorly described *Pseudomonas "reactans"*. Anaccurate identification of *P. tolaasii* was carried out by sequencing 16SrDNA gene. Phenotypically the isolates were heterogeneous, which further hindered their correct identification. Regarding *P. "reactans*", this bacterial name is not regarded as a valid name and there is no type strain. In this work we have named as *P. reactans* type those isolates that induce the precipitation of tolaasin. This would suggest that we are not dealing with one species but a group of bacteria. Both, *P. tolaasii* and *P. reactans* were shown to be pathogenic in the assays performed, so the aetiology of the disease can be manifold.The incidence of blotch disease in Spanish mushroom crops was evaluated to affect up 15% of the total yield, with the highest losses detected for the second flush. The development of the disease wasassociated with high relative air humidity registered on the irrigation day closest to the harvesting of the second flush, which may lead to condensation on the caps as soon as the air temperature changesif there is no adequate ventilation. Modifying the environmental conditions by increasing the temperature prior to irrigation and moderating the manoeuvres of the drying processhelped control bacterial blotch disease.

1. Introduction

The most common cultivated mushroom, *Agaricusbisporus* (Lange) Imbach, is susceptible to a variety of bacterial diseases, such as soft rot, internal stipe necrosis, drippy gills, and ginger and brown blotch diseases, which mainly affect the quality of the mushrooms (Fermor, 1987; Reyes et al., 2004; Fletcher and Gaze 2008; González et al., 2009, 2012). Of these, brown blotch disease, which is characterised by superficial, shining brown-stained lesions, irregular and often sunken, on the mushroom caps, is the most common and harmful (Munsch et al., 1991; Munsch and Alatossava, 2002). Under favourable environmental conditions the lesions, initially small and separate, may coalesce and affect large areas of the pileus (Iacobellis, 2011). The browning affects only the external layers of the cap tissues, and is restricted to 2–3 mm below the surface of the cap (Soler-Rivas, 1998). Affected mushrooms became sticky to the touch (Fletcher and Gaze, 2008). Traditionally *Pseudomonas tolaasii* Paine has been considered as the main causal agent of the disease. In suitable conditions or when stimulated by exudates from the mushroom caps, these bacteria produce tolaasin as an end product of its metabolism. This substance, at a sufficient concentration, can be toxic for the *Agaricus* mycelium, and results in a (generally) superficial lysis of cap tissues (Gill, 1995). Moreover, other species of bacteria have also been described as causing blotch diseases involving various discolorations and appear to participate in the expression of disease symptoms (Wong and Preece, 1982; Godfrey et al., 2001; Munsch and Alatossava, 2002; Lo Cantore and Iacobellis, 2004; Iacobellis, 2011; Milijasevic-Marcic et al., 2016; Van der Wolf et al., 2016). Of particular note in this respect is *P. constantini*, described by Munsch and Alatossava (2002) as responsible for this disease, and, especially, *P. reactans*, a bacterium that has been traditionally associated with discoloured and sunken lesions on mushroom sporophores (Largeteau and Savoie, 2010). However, to the best of our knowledge, *P. reactans* has not been described officially and there are no type strains includedin any collection.For this reason, in this work we include as "reactans type" those fluorescent pseudomonas that lead to the precipitation of tolaasin as a result of the production of lipodepsipeptide, known as the White Line-Inducing Principle (WLIP). This group of bacteria showed reduced virulence in *A. bisporus* tissue compared with *P. tolaasii* strains (Lo Cantore and Iacobellis, 2014).

Pseudomonas bacteria are present in a variety of organic wastes –

* Corresponding author. Centro de Investigación, Experimentación y Servicios del Champiñón (CIES), c/Peñicas s/n. E–16220, Quintanar del Rey, Cuenca, Spain.
E-mail addresses: mjnavarro.cies@dipucuenca.es, mjnl111@hotmail.com (M.J. Navarro), fjgea.cies@dipucuenca.es (F.J. Gea), anagf@serida.org (A.J. González).

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compost, peat, etc.- and are easily carried by sciarid and phorid flies, mites, dust particles, pickers and their tools (Munsch et al., 1991). The manifestation of blotch can vary from small lesions occurring on a few mushrooms (5% of total yield) to a severe incidence whereby all the mushrooms on a bed will be severely affected (Miller and Spear, 1995). When severe, an outbreak of bacterial blotch may result in losses of mushroom crops of up to 10–25% due to the loss in quality and hence market value (Fermor et al., 1991). It is also considered a post-harvest disease of mushroom, because further losses may occur after the mushrooms have been harvested (Fermor et al., 1991; Miller and Spear, 1995; Wells et al., 1996). The development of the disease is induced by a high relative air humidity, which can lead to condensation on the caps as soon as the air temperature changes without any adequate ventilation (Wuest, 1971; Munsch et al., 1991). Dew point is the temperature at which the water vapour in the air starts to condense or sweat on cold glass. If the temperature of the mushroom cap is lower than the dew point temperature of the air, water vapour will condense on the surface of the cap. Dew point temperature is a concern during cropping particularly when relative humidity is high and the temperature is changing (Lomax, 2007). Once the disease has become established in the crop, watering will scatter the bacteria (Fletcher and Gaze, 2008).

Agaricus strains exhibit differences in susceptibility to bacterial blotch (Rama et al., 1995; Olivier et al., 1997; Mamoun et al., 1999, 2000; Soler-Rivas et al., 1999), so breeding for resistance to bacterial disease is one possible control strategy (Fermor, 1987). There are also some papers on the biological control of bacterial blotch using antagonistic bacteria (Bashan and Okon, 1981; Miller and Spear, 1995; Munsch and Olivier, 1995; Largeteau and Savoie, 2010; Tajalipour et al., 2014), although the use of the phages has been restricted to high risk periods. More recent works proposed the possibility of the phytochemical control of the disease, using several plant extracts, but the results have been variable (Sokovic and van Griensven, 2006; Malpani et al., 2012). Nowadays, the two most widely used methods by mushroom growers for controlling the disease are the application of regular chlorinated water drenches to the casing layer, and manipulation of the environmental conditions (Diamantopoulou et al., 2000; Lomax, 2007; Fletcher and Gaze, 2008).

This study has the following objectives: a) to identify the causal agent of bacterial blotch in Castilla-La Mancha mushroom crops; b) to evaluate the incidence of bacterial blotch disease in commercial mushroom crops from Castilla-La Mancha and c) to suggest how environmental conditions can be managed to help control bacterial blotch disease.

2. Materials and methods

2.1. Identification of bacterial blotch causal agent

Twenty two samples of cultivated *A. bisporus* with brown blotch symptoms, harvested in a mushroom growing farm (MERCAJÚCAR Soc. Coop., Castilla-La Mancha, Spain) and collected between 2009 and 2011, were analyzed by dilaceration tissue in sterile distilled water and subsequent plating on KB medium (King et al., 1954). The plates were incubated at 25 °C for 48 h, and 127 bacterial isolates were obtained.

The isolates were identified by phenotypic tests such as gram, fluorescence under UV light, LOPAT scheme (Lelliott et al., 1966) and oxidation/fermentation of glucose (Hugh and Leifson, 1953). Bacteria corresponding to groups Va and IVb of the LOPAT scheme were selected. In order to differentiate *P. tolaasii*, a WLIP test was performed with the LPPA 540 and 542 strains, corresponding to bacteria named as *P. reactans* and *P. tolaasii*, respectively, as controls.

The isolates were characterised according to their phenotypic features such as hydrolysis of esculin, gelatin, caseine and tween 80, the use of D-tartrate, L-Lactate, trigonelline, betaine, quinate, homoserine, adonitol, sucrose, xylose and lactose as carbon source in Ayers medium (Lelliott and Stead, 1987), and the use of mannitol, sorbitol, erythritol and m-inositol in Hellmers medium (Jansing and Rudolph, 1990).

Amplification of 16S rDNA gene was carried out using the pA and pH primers (Edwards et al., 1989) in a MJ Research PTC 100 thermocycler (Waltham, Mass., USA), using 2.5 µl of overnight bacterial culture grown on YPG broth (ca. 10^8cfu/mL), 0.3 µM of each primer, a 0.2 mM of each deoxynucleoside triphosphate, 2 U of DyNAZyme II DNA polymerase (FinnzymesOy, Espoo, Finland), and 5 µl of the supplier's reaction buffer. The volume was made up to 50 µl with sterile double-distilled water. After a 3-min denaturation step at 94 °C, the reaction mixture was run through 30 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 90 s, followed by a final step at 72 °C for 10 min. The ca. 1500-bp PCR products were purified with the Ultraclean PCR clean up DNA purification kit (MO-BIO, Inc., USA) as recommended by the manufacturer, and used directly for sequencing analysis.The nucleotide sequence of 16S rDNA gene was sequenced by Secugen (Madrid, Spain), and a comparison with sequences deposited in the GenBank DNA database was made using the BLAST algorithm (Altschul et al., 1990).The identities of the isolates were determined on the basis of the highest score. The sequences were aligned with ClustalW Multiple Alignment (Thompson et al., 1994). Phylogenetic trees were obtained using the neighbour-joining method with the Kimura two-parameter model, with 1000 bootstrap replications using Mega 6 software (Tamura et al., 2013).

Bacterial suspensions grown for 16 h in yeast peptone glucose broth (ca. 10^8cfu/mL) were inoculated into mushroom caps (Inglis et al., 1996) of *A. bisporus* by puncture with toothpicks, and then incubated at 22° C in glass dishes. Sterilized distilled water was used as control. Assays were conducted twice and the results were recorded over a period of ten days. From de symptomatic caps, bacteria sharing the characteristics of the inoculated isolates were recovered, for fulfilling Koch's postulates.

2.2. Incidence of bacterial blotch disease in a commercial mushroom growing farm and management of the environmental conditions

The study was carried out between 2009 and 2011 in a mushroom growing farm (MERCAJÚCAR Soc. Coop., Castilla-La Mancha, Spain) that was suffering high crop losses due to bacterial blotch. Five mushroom crops were monitored during the periods when the disease was most obvious (autumn and spring). The information gathered during the first two crops (autumn09 and spring10) was used to evaluate disease incidence and to study the environmental factors possibly favouring bacterial proliferation. The other three mushroom crops (autumn10, spring11 and autumn11) were used to contrast the effectiveness of the proposed environmental control measures.

The growing rooms were equipped with a five-level shelf and a climate computer system. Dataloggers and evaporimeters were placed 5 cm above the surface of each shelf in order to record the environmental temperature and daily evaporation rate for each level. The days and volume of water used to irrigate the crops were recorded during the harvesting period.

Commercial mushroom phase II compost spawned at 1% (Amycel XXX) was used. On day 0 of the cropping cycle, spawn-run compost was cased with a layer of a casing soil made with mineral soil + Sphagnum peat 4:1 (v/v). In each of the five shelves (shelf 1: at the bottom; shelf 5: at the top), an area of 2.3 m^2 was delimited, where all the mushrooms formed during the first three flushes were picked daily. These were classified as healthy or affected by bacterial blotch, and the weight per m^2 of each group was recorded. Samples of the diseased mushrooms were sent to SERIDA (Villaviciosa, Asturias, Spain) for identification of the pathogen involved. Disease incidence was recorded as a percentage value, based on the ratio of the weight of diseased sporophores to the total yield (healthy + diseased mushroom) (Fermor et al., 1991; Geels, 1995).

In order to compare single crops, statistical analyses of total